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AMPA Receptor-Dependent Clustering of Synaptic NMDA Receptors Is Mediated by Stargazin and NR2A/B in Spinal Neurons and Hippocampal Interneurons

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Summary

Under standard conditions, cultured ventral spinal neurons cluster AMPA- but not NMDA-type glutamate receptors at excitatory synapses on their dendritic shafts in spite of abundant expression of the ubiquitous NMDA receptor subunit NR1. We demonstrate here that the NMDA receptor subunits NR2A and NR2B are not routinely expressed in cultured spinal neurons and that transfection with NR2A or NR2B reconstitutes the synaptic targeting of NMDA receptors and confers on exogenous application of the immediate early gene product Narp the ability to cluster both AMPA and NMDA receptors. The use of dominant-negative mutants of GluR2 further showed that the synaptic targeting of NMDA receptors is dependent on the presence of synaptic AMPA receptors and that synaptic AMPA and NMDA receptors are linked by Stargazin and a MAGUK protein. This system of AMPA receptor-dependent synaptic NMDA receptor localization was preserved in hippocampal interneurons but reversed in hippocampal pyramidal neurons.

Introduction

The molecular mechanisms governing the formation of excitatory synapses in the central nervous system are the subject of intense and increasingly detailed investigation (Goda and Davis, 2003). Previously, we proposed that the mechanisms governing the formation of glutamatergic synapses on neurons that receive excitatory synapses directly onto their dendritic shafts, such as spinal cord neurons and hippocampal interneurons, differed significantly from those that govern the formation of excitatory synapses on neurons that have dendritic spines, such as hippocampal pyramidal neurons (Mi et al., 2002). These mechanistic differences mirror the distribution of the immediate early gene product Narp, which has been proposed to play a role in aggregating the AMPA class of glutamate receptors at excitatory synapses on dendritic shafts (O'Brien et al., 1999, 2002). The manner in which NMDA receptors become aggregated at excitatory synapses on dendritic shafts requires an explanation other than a direct interaction with Narp, since no such interaction has been demonstrated.

Much interest and research has gone into the coupling of NMDA- and AMPA-type glutamate receptors at excitatory synapses since the "silent synapse" theory of synaptic plasticity was proposed in 1995 (Liao et al., 1995; Isaac et al., 1995). This theory requires a regulated coupling between NMDA- and AMPA-type glutamate receptors on dendritic spines. Recently, work in hippocampal pyramidal neurons has shown that NMDA receptors may be targeted to excitatory synapses through interactions involving the Ephrin/EphR (Gerlai, 2001; Cutforth and Harrison 2002) or neuroligin/neurexin systems (Scheiffele et al., 2000). Subsequent interactions between NMDA receptors and the transmembrane coupling molecule stargazin then draws AMPA receptors to these synapses. Stargazin is a critical link because it interacts with AMPA-type glutamate receptors via one domain and interacts with the MAGUK class of cytoplasmic anchoring molecules, such as PSD-95, at another site. The interaction between MAGUK proteins and stargazin occurs through the C-terminal tail of stargazin, which contains a PDZ binding site (Chen et al., 2000; Schnell et al., 2002; Tomita et al., 2003). Similar sites are also present on the C terminus of NR2 subunits, which also interact with MAGUK proteins.

A challenge to the central role of NMDA receptors in synaptic glutamate receptor targeting was first revealed in animals without NMDA receptors that have retained synaptic AMPA receptors (Tsien et al., 1996), as well as in work recently described in hippocampal interneurons (Nyiri et al., 2003), which shows significant variability in synaptic NMDA receptor clustering in spite of a more uniform synaptic AMPA receptor expression.

In cultured spinal neurons, which have a robust, Gaussian distribution of AMPA receptors at excitatory synapses, little synaptic NMDA receptor clustering is noted (O'Brien et al., 1997, 1998). The preferential clustering of AMPA receptors at spinal synapses in vitro occurs in spite of robust expression of the ubiquitous NMDA receptor subunit NR1 (Mi et al., 2002) and a chemosensitivity to exogenous NMDA that is equivalent to that of cultured hippocampal neurons that have synaptic NMDA receptors (O'Brien et al., 1997). Chimeric cultures of hippocampal and spinal neurons indicate that the lack of synaptic NMDA receptor clustering in spinal neurons is due to a lack of expression of one or more proteins by the postsynaptic spinal neurons rather than to the lack of a necessary presynaptic organizing molecule (Mi et al., 2002).

In this paper, we show that cultured spinal neurons under basal conditions lack the expression of the NMDA

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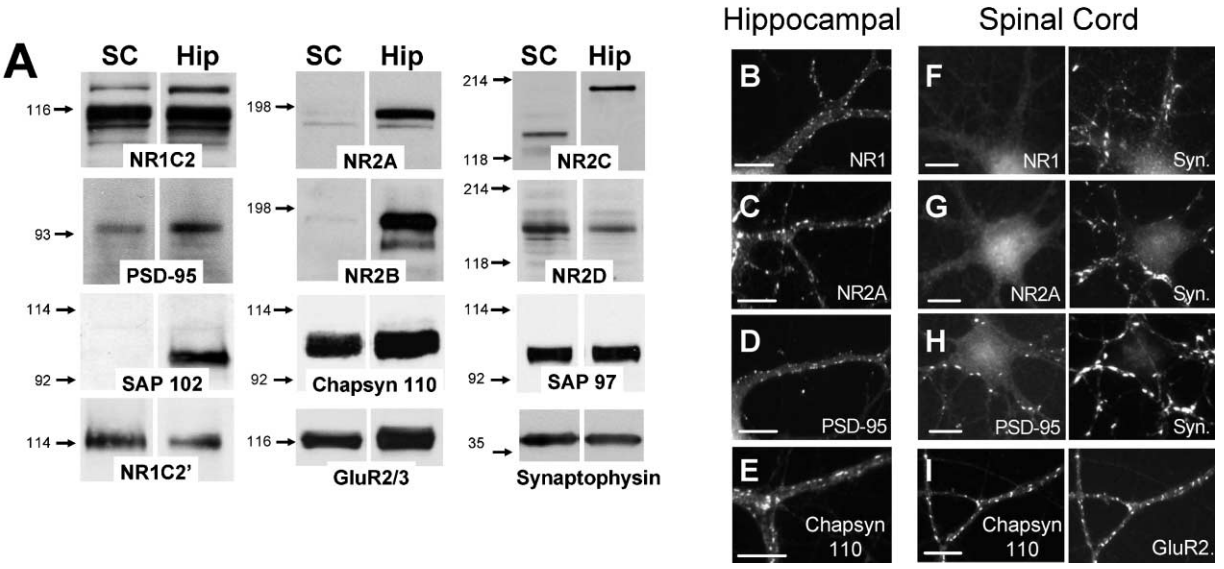

Figure 1. NMDA Receptor Clustering in Ventral Spinal Neurons

(A) High-density cultures of hippocampal or ventral spinal neurons were grown at a similar density for 12 days in vitro. Cultures were solublized in equal volumes of M-Per plus protease inhibitors (200,000 cells/ml) and run on SDS PAGE and probed with the indicated antibodies. All experiments were done in triplicate and yielded similar results. In (B)–(I), 12 day cultures of hippocampal and ventral spinal neurons were immunostained with the indicated antibodies showing (synaptic) clusters of NR1 (B) and NR2A (C) in hippocampal neurons but not in spinal neurons (F and G). PSD-95 (D and H) and Chapsyn-110 (E and I) showed synaptic clustering in both (calibration bar, 10 μm). The high molecular weight band seen with the NR2C antibody in hippocampal cultures represents a cross-reacting NR2A or NR2B epitope and is absent in spinal cultures which lack NR2A or NR2B.

receptor subunits NR2A and NR2B. Synaptic targeting of endogenous NR1 in spinal neurons can be reconstituted by transfection with either NR2A or NR2B and requires a linkage to preexisting synaptic AMPA receptors, independent of additional presynaptic targeting molecules. This linkage requires stargazin and a MAGUK protein, although additional NR2-mediated interactions are also likely. This mechanism of synaptic targeting of NMDA receptors in spinal neurons is preserved in the excitatory synapses on the dendritic shafts of hippocampal interneurons but differs from that present in hippocampal pyramidal neurons, expanding the molecular differences between excitatory synapses on neurons with or without dendritic spines.

Results

NR2A and NR2B Target NMDA Receptors to Synapses

Previous work in our lab indicated that the lack of synaptic NMDA receptor clustering in spinal neurons was due to the lack of expression of a crucial protein in postsynaptic spinal dendrites (Mi et al., 2002). We began our current investigation by examining which postsynaptic components, thought to be important for synaptic NMDA receptor clustering in other systems, might be absent in ventral spinal neurons. We used immunoblots of spinal and hippocampal cultures to look for known components of central postsynaptic densities (Sheng, 2001). Two important, reproducible deficiencies in cultured spinal neurons compared to hippocampal neurons were the severe reduction in the expression of the NMDA receptor subunits NR2A and NR2B (Figure 1A). This difference in expression of NR2A and NR2B was seen either with antibodies to the interior portion of the C-terminal tail (shown) or with antibodies to the extreme C terminus (not shown). The deficit in NR2A and NR2B expression and the lack of synaptic NMDA receptors in spinal neurons was present both at 1 week and 2 weeks in vitro (Figure 1F; see also Figure 5), while in hippocampal neurons, synaptic NMDA receptors (Figure 1B) and NR2A and NR2B expression (Figure 1A) was easily detectable at 7 days and fully expressed by 10 days in vitro. Also noted in these studies was a relative deficiency of PSD-95, a lack of SAP-102, and an excess expression of NR2C in spinal neurons. Other proteins tested, aside from those shown in Figure 1, that showed no significant differential expression between spinal and hippocampal cultures included GRIP1 and GRIP2, Shank, NSF, and PICK1.

Immunostaining of cultured ventral spinal neurons with antibodies to NR1 (Figure 1F; S3C11-pan NR1) and all four NR2 receptor subunits (Figure 1G demonstrates representative NR2A immunostaining) showed almost no clustering of NMDA receptor subunits in spinal neurons after 1 or 2 weeks in vitro and minimal synaptic localization. Surprisingly, PSD-95 (Figure 1H) and Chapsyn-110 (Figure 1I) showed excellent synaptic localization in ventral spinal neurons in the absence of synaptic NMDA receptor clustering. No synaptic staining was seen with antibodies to SAP-102 or SAP-97 in ventral spinal neurons (data not shown). In contrast, in cultured hippocampal neurons, clustered immunostaining was seen with the NR1 (Figure 1B) and all NR2 subunit-specific antibodies (Figure 1C shows representative staining for NR2A) with the exception of the NR2C anti-

Table 1. Transfection of Ventral Spinal Neurons with NR2A or NR2B Induces Synaptic Clustering of NMDA Receptors

A

	Synaptic Clusters/Cell	
Transfected with:	NR1	GluR2
Control	1.5 ± 0.5	11.9 ± 3.5
NR1-1A (C2)	1.1 ± 0.3	13.1 ± 3.1
NR1-4A (C2′)	1.6 ± 0.5	10.6 ± 2.2
NR2A	7.8 ± 2.3**	13.6 ± 3.0
NR2A plus APV/CNQX	6.9 ± 1.7**	11.1 ± 2.8
NR2B	8.9 ± 4.1**	10.3 ± 2.1
PSD-95	2.2 ± 0.7	9.9 ± 1.8
SAP-102	2.6 ± 0.9	8.8 ± 2.1
NR2AΔ10	2.7 ± 0.9	9.6 ± 2.8
NR2C	1.6 ± 0.6	15.0 ± 4.2
NR2D	2.6 ± 1.1	14.2 ± 3.4

B

Transfected with:	Synaptic NR1 Clusters	Mean Synaptic NR1 Fluorescence Intensity
Control	2.2 ± 1.1	—
NR2A	12.8 ± 3.2**	2291 ± 626
NR2A/D6	8.9 ± 2.3**	1004 ± 436***
NR2A/A6	3.6 ± 1.5	—
NR2A/Δ6	2.9 ± 1.6	—

In panel (A), spinal neurons, grown for 3 days in vitro, were transfected with the indicated constructs (Control is pCMV-LacZ) plus a small amount of HcRed (or eGFP) to identify the transfected cells, replated, and grown for 4 or 5 days, at which time they were fixed and stained for synaptophysin plus GluR2 or NR1. The numbers expressed are the mean ± SD of the mean of four separate experiments, except for NR2C and NR2D, where three transfections were performed. All constructs except NR2C and NR2D were transfected concurrently. NR2C and NR2D were transfected in a separate series of experiments with a concomitant positive control (NR2B) which showed the expected induction of synaptic NR1 clusters (11.2 ± 2.9 per cell). For each experiment, 20 to 25 consecutive, randomly selected HcRed (or eGFP) positive neurons were chosen, and the number of synaptophysin-associated GluR2 or NR1 clusters on the selected neuron was counted in one field (cell centered) at 100×. The slides were blinded before counting. In panel (B), a second set of cultures were transfected with the indicated constructs, and the number of synaptic NR1 clusters was determined along with the mean synaptic NR1 fluorescence intensity at synapses deemed immunopositive for NR1. **$p < 0.001$ compared with control in a paired comparison; ***$p < 0.02$ compared to NR2A.

body, which did not show clustered staining in either hippocampal or spinal neurons. The hippocampal NR2A and NR2B synaptic accumulation was seen in the vast majority of neurons as soon as synaptic NR1 was detected. Excellent synaptic localization was also seen in hippocampal neurons with antibodies to PSD-95 (Figure 1D) and Chapsyn-110 (Figure 1E), but not with antibodies to SAP-102 and SAP-97 (data not shown). At this stage in culture, nearly all NMDA and PSD/Chapsyn receptor clusters in spinal and hippocampal neurons are associated with presynaptic synaptophysin staining, indicating that they are synaptic (Liao et al., 1999; Mi et al., 2002).

Given the deficiency of NR2A and NR2B expression in cultured spinal neurons and their purported role in synaptic NMDA receptor localization (Barria and Malinow, 2002), we transfected NR2B or NR2A into ventral spinal neurons 72 hr after plating, along with a small amount of a construct encoding a fluorescent protein to identify transfected cells, replated them onto glial cells, and allowed them to grow for an additional 4–5 days. As shown in Table 1 and in the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/], expression of either NR2A or NR2B in ventral spinal neurons was able to reconstitute synaptic NMDA receptor accumulation as assayed by synaptic NR1 clustering. Quantitatively, the mean number of synaptic NR1 clusters increased nearly 5-fold (Table 1) with either NR2A or NR2B transfection, while the number of synaptic GluR2 clusters was unchanged. Transfection with a mutant of NR2A (NR2AΔ10) that lacks the last ten amino acids of its intracellular C terminus, the portion responsible for binding to the PDZ domains of PSD-95 and other MAGUK proteins (Hung and Sheng, 2002), significantly reduced the synaptic targeting of NR1 (Table 1). The NR2A- or NR2B-induced accumulation of synaptic NMDA receptors occurred in the presence or absence of synaptic activity, as APV (0.5 mM) plus CNQX (10 μM) did not affect the NR2A-induced accumulation of NR1. Overexpression of the NMDA subunits NR1-1A, containing the C2 cassette; NR1-4A, containing the C2′ cassette; NR2C; or NR2D in ventral spinal neurons had no effect on the synaptic distribution of NR1, nor did overexpression of PSD-95 or SAP-102.

In order to ensure that NR2A and NR2B were increasing the surface, synaptic pool of NMDA receptors rather than simply aggregating a subsurface synaptic pool, we transfected NR1-1A, containing an extracellular *myc* epitope, along with NR2A and -B and looked at live surface staining. As shown in Figure 2A and in the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/], when *myc*-NR1 is transfected into spinal neurons alone there is a significant extrasynaptic surface accumulation, typical of the extrasynaptic AMPA receptors seen in these same cultures (Mammen et al., 1997). Like extrasynaptic AMPA receptor clusters (Mammen et al.,

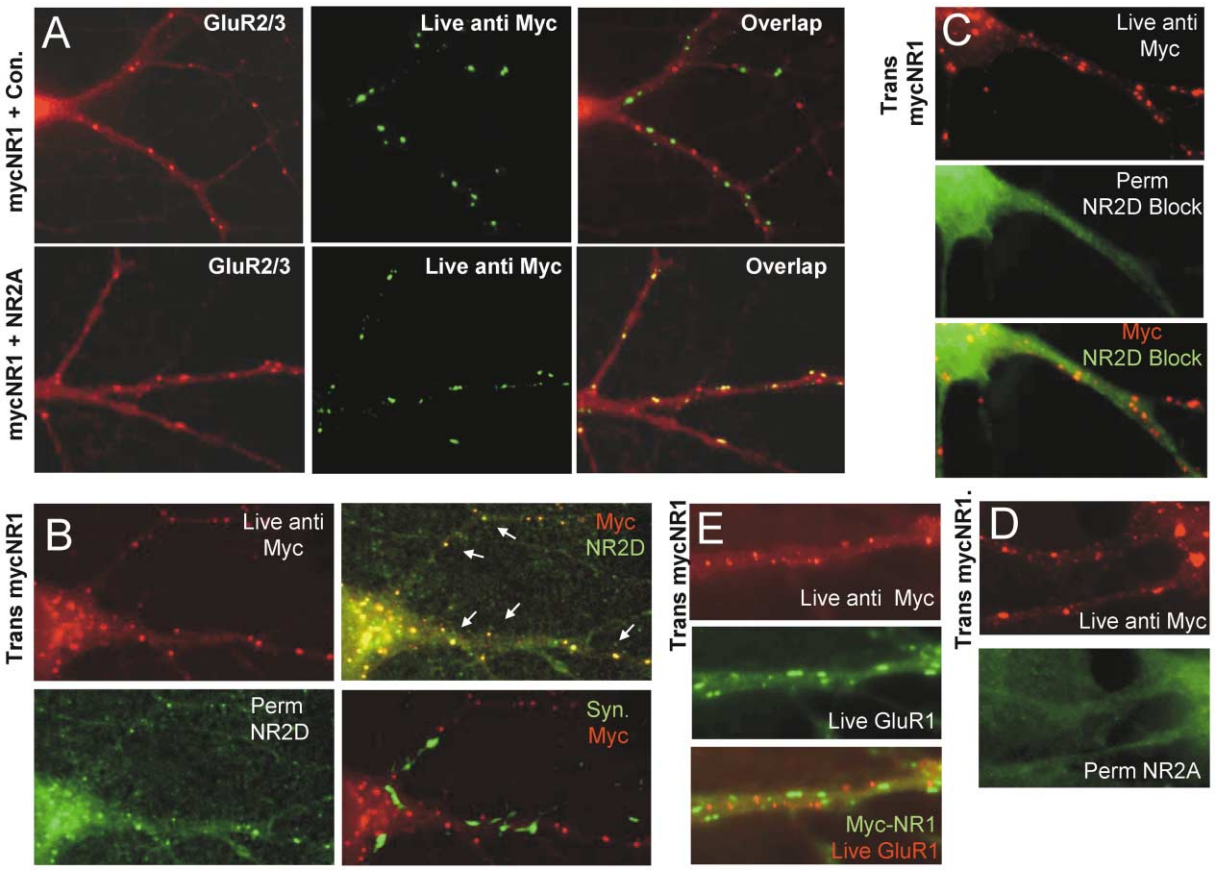

Figure 2. Effect of NR2A on the Surface Expression of NR1

Ventral spinal neurons were transfected either with *myc*-tagged NR1-1A plus untagged NR2A or with *myc*-tagged NR1-1A plus control. Following transfection, neurons were stained live with anti-*myc* to identify surface NR1 and then fixed, permeablized, and stained with anti-GluR2/3 (A) to identify excitatory synapses. In (B) and (D), after transfecting with *myc*-NR1, neurons were stained live with anti-*myc* and then fixed, permeablized, and stained for synaptophysin (Goat) plus either NR2D or NR2A (rabbit), respectively. In (C), the NR2D antibody was blocked with NR2D transfected HEK293 extract as described in Experimental Procedures. In (E), live staining of *myc*-NR1 transfected neurons was carried out with both anti-*myc* and anti-GluR1.

1997), the punctate appearance of extrasynaptic NR1 is a useful artifact of the bivalent anti-*myc* antibody and is not seen when Fab fragments are used. Simultaneously labeling the surface extrasynaptic *myc*-NR1 subunits with the mouse monoclonal anti-*myc* antibody revealed colocalization with NR2D (Figure 2B, arrows) in greater than 80% of the extrasynaptic *myc*-NR1 clusters seen in a series of 34 transfected neurons from two platings (546 of 627 extrasynaptic *myc*-NR1 clusters), implying that they are part of a receptor complex. Little colocalization of extrasynaptic *myc*-NR1 was seen with either NR2A (Figure 2D) or NR2B (data not shown), permeablized GluR2/3 (Figure 2A), or surface GluR1 (Figure 2E). In contrast, when *myc*-NR1 is transfected with untagged NR2A, there is a significant synaptic accumulation of *myc*-NR1 (Figure 2A) and a decrease in extrasynaptic clusters (see the Supplemental Data). The surface synaptic accumulation of *myc*-NR1 was also seen in NR2A-transfected neurons stained live with an Fab anti-*myc* antibody (10.6 $\pm$ 5.9 surface synaptic *myc*-NR1 clusters per neuron [*myc*-NR1 + NR2A] versus 1.4 $\pm$ 1.9 [*myc*-NR1 alone]; n = 28 and 25 neurons, respectively), eliminating the possibility that the bivalent antibody induced the synaptic clusters. The same result was seen when *myc*-NR1-1A was cotransfected with NR2B but not with NR2C, NR2D, or NR1-4A (see the Supplemental Data). These results suggest that extrasynaptic NMDA receptors make it to the surface of dendrites and are part of a multimeric complex with NR2D. When NR2A or NR2B are present, the receptor complex localizes to the synapse. Further studies using immunoprecipitation in cultured spinal neurons support this model (see below).

Are MAGUK Interactions Necessary and Sufficient for Synaptic NMDA Receptor Targeting?

The results presented above show that the MAGUK-interacting portions of NR2A and NR2B are necessary for the synaptic targeting of NMDA receptors. The question of why NR2C and NR2D, which have C-terminal amino acid sequences similar (although not identical) to NR2A and NR2B, are not sufficient to induce synaptic clustering of NMDA receptors was examined. First we examined the immunoprecipitation of soluble, *myc*-tagged, C-terminal tails of NR2A and NR2D with PSD-95 in HEK293 cells (Figure 3A). We coexpressed a 30 kDa C-terminal fragment of NR2A along with a 16 kDa C-terminal fragment of NR2D and full-length PSD-95.

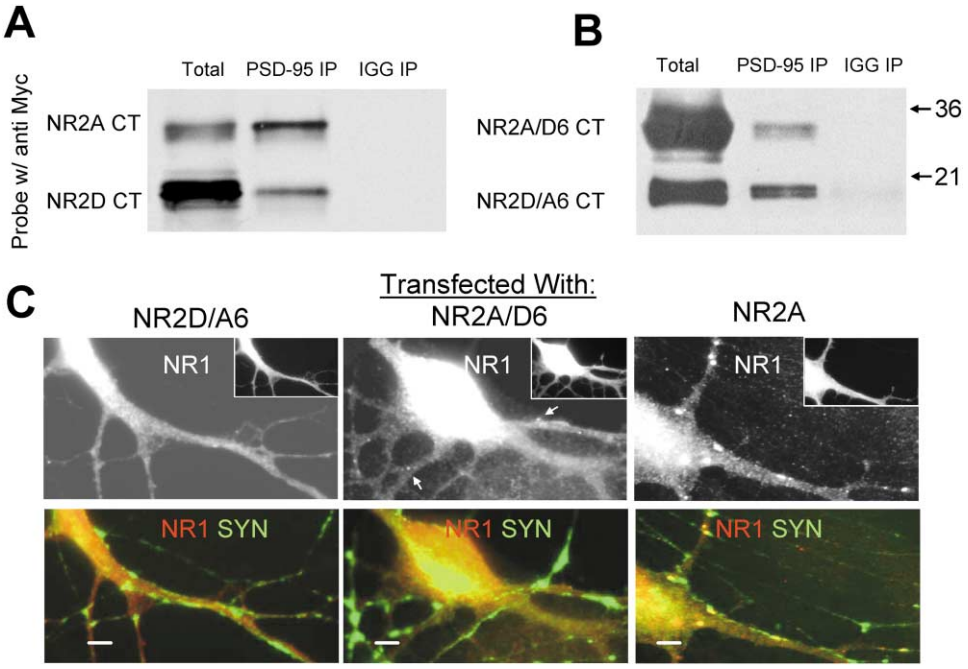

Figure 3. The Mechanism of NR2A-Induced Synaptic Clustering of NMDA Receptors

HEK293 cells were transfected with N-terminal *myc*-tagged versions of the C terminus of NR2A (30 kDa) or NR2D (16 kDa) with a 2:1 DNA excess of NR2DCT along with PSD-95. PSD-95 was immunoprecipitated with mouse anti-PSD-95, and the total input and the immunoprecipitate probed with rabbit anti-*myc*. Total represents 1/20 of the total material, while the IP represents 1/2 of the IP material. In (B), chimeric NR2A and NR2D molecules are simultaneously coexpressed with PSD-95 in HEK293 cells. In this case, a 2:1 DNA excess of NR2A/D6 was used compared with NR2D/A6. In these chimeras, the last six amino acids were switched. In (C), neurons transfected with the different NR2 constructs show representative synaptic NR1 distributions. Note the small synaptic clusters seen with NR2A/D6 (arrows) compared with native NR2A. Scale bar, 4 μm.

Both NR2 fragments contained N-terminal *myc*-tags. Total NR2D was expressed in excess compared with NR2A by manipulating DNA concentrations. Immunoprecipitation of PSD-95 resulted in the coimmunoprecipitation of both the NR2A and NR2D C-terminal fragments, but a far greater excess of NR2A was immunoprecipitated even though it was underexpressed compared to NR2D. When the terminal six amino acids of NR2A and -D were switched, creating chimeric C-terminal fragments, the interaction between NR2A, NR2D, and PSD-95 was reversed (Figure 3B). This implies that the C terminus of NR2D is less capable of interacting with PSD-95 than that of NR2A and may explain part of the inability of NR2C and NR2D to induce the synaptic targeting of NMDA receptors. A similar result was seen with Chapsyn-110 (data not shown). Both PSD-95 and Chapsyn-110 coimmunoprecipitations with NR2 C-terminal fragments from HEK293 cells were performed twice with similar results.

In a second set of experiments designed to test whether the MAGUK binding domain of NR2A and -B was sufficient for synaptic targeting, we transfected ventral spinal neurons with chimeric NR2 subunits. The first, NR2A/D6, consisted of full-length NR2A whose final six amino acids were switched to that of NR2D, while NR2D/A6 consisted of full-length NR2D whose final six amino acids were switched to that of NR2A. These constructs, along with GFP, were transfected into spinal neurons, and the transfected neurons were assayed for the synaptic clustering of NR1. As shown in Figure 3C and Table 1 (panel B), the NR2D/A6 chimera showed almost no ability to induce the formation of synaptic NR1 clusters. In contrast, NR2A/D6 did induce synaptic clustering of NR1, but at a reduced rate compared with native NR2A and with a smaller mean synaptic receptor cluster size compared to native NR2A. NR2AΔ6, an NR2A mutant lacking its terminal six amino acids, showed no ability to induce synaptic clusters of NR1, like the previously described deletion mutant NR2AΔ10.

These data demonstrate that the C-terminal PDZ binding domain of NR2A is necessary for the synaptic clustering of NMDA receptors and is more potent than a similar motif present on NR2D. However, the data also suggests that this PDZ binding motif is not sufficient for synaptic targeting, suggesting that an additional motif present on NR2A and absent from NR2D is important for synaptic targeting. Direct interactions between NR2A and Narp, a potential AMPA receptor organizing molecule that interacts with the N terminus of AMPA receptors, have not been demonstrated (see below), but this mode of interaction between NMDA receptors and another extracellular organizing molecule(s) may be relevant (see Discussion).

Narp Clusters NMDA Receptors in NR2A Transfected Cells

In addition to its effect on the synaptic targeting of NMDA receptors, transfection of ventral spinal neurons with NR2A conferred on them a phenotype whereby exogenous Narp could induce clusters of NMDA recep-

Table 2. Dominant-Negative Mutants of GluR2 and Stargazin Disrupt PSD-95 and NR2A-Mediated Synaptic Clustering of NMDA Receptors in Spinal Neurons

A

Transfected with:	Synaptic PSD-95 Cluster	Synaptic Chapsyn-110	Synaptic GluR2 Clusters	Synaptic GluR1 Clusters	Synaptic Stargazin Clusters (n = 2)
Con (n = 3)	13.1 ± 3.7	12.3 ± 3.1	12.8 ± 2.5	9.7 ± 2.8	14.2 ± 4.3
GluR2CT (n = 3)	4.3 ± 1.9**	3.2 ± 2.2**	3.7 ± 1.4**	4.2 ± 1.1**	4.3 ± 2.1**
GluR1CT (n = 3)	11.7 ± 2.6	14.2 ± 3.8	13.7 ± 3.2	11.3 ± 3.3	12.9 ± 2.7
NR2ACT (n = 3)	10.9 ± 2.8	11.0 ± 3.5	12.3 ± 4.0		
StargazinΔC (n = 3)	4.7 ± 1.9**	4.0 ± 1.6**	9.9 ± 2.7	8.6 ± 2.0	—
StargazinCT (n = 3)	3.2 ± 1.3**		11.5 ± 3.0		
StargazinCTΔ10 (n = 3)	9.9 ± 2.1		14.1 ± 2.8		
Stargazin (n = 3)	14.3 ± 3.9		15.6 ± 2.3**		

B

Transfected with:	NR1 Clusters	GluR2 Clusters
NR2A + Con. (n = 6)	7.3 ± 2.0	10.9 ± 2.1
NR2A + GluR2CT (n = 3)	2.8 ± 1.3**	4.1 ± 1.3**
NR2A + GluR1CT (n = 3)	8.5 ± 2.6	9.9 ± 3.2
NR2A + stargazinΔC (n = 3)	2.1 ± 0.6**	9.8 ± 2.1
NR2A + stargazinCT (n = 3)	3.6 ± 1.5***	13.9 ± 3.3
NR2A + stargazinCTΔ10 (n = 3)	6.1 ± 1.4	10.7 ± 2.0
NR2A + stargazin (n = 3)	6.4 ± 1.7	12.8 ± 2.3

Ventral spinal neurons were transfected with the indicated constructs plus a small amount of HcRed (or eGFP) to identify the transfected cells, replated, and grown for 4–5 days, at which time they were fixed and stained for PSD-95, GluR2, GluR1, Chapsyn-110, Stargazin, or NR1 along with synaptophysin. The numbers expressed are the mean ± SD of the mean of three separate experiments, except for the NR2A plus Control, which is the result of six experiments, and the stargazin, which is the result of two experiments. For each experiment, 20 to 25 consecutive, randomly selected HcRed/GFP-positive neurons were chosen, and the number of synaptic GluR1, GluR2, PSD-95, stargazin, chapsyn, or NR1 clusters counted in one field (cell centered) at 100×. The slides were blinded before counting. GluR1 clusters were determined in cells stained live and thus represent surface clusters. The GluR1 synaptic clusters were counted only in neurons with at least one synaptic GluR1 cluster, as is our usual routine, given that 40% of cultured spinal neurons do not express GluR1. **$p < 0.01$ compared with control in a paired comparison; ***$p < 0.05$ compared with control in a paired comparison.

tors at sites of contact between Narp-expressing COS cells and the dendrites of spinal neurons (see the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/]). Such a phenotype has already been observed in untransfected hippocampal interneurons (Mi et al., 2002).

Synaptic NMDA Receptor Targeting Is Linked to AMPA Receptor Targeting

Since previous work has shown that Narp does not interact with NMDA receptors on spinal neurons or transfected HEK293 cells (O'Brien et al., 1999, 2002), it is likely that the NR2A-mediated aggregation of NMDA receptors seen in transfected spinal neurons is due to the coaggregation of NMDA receptors with an unknown Narp-interacting postsynaptic protein or to the cytoplasmic coupling of NMDA receptors and AMPA receptors. To test whether the postsynaptic aggregating machinery of AMPA and NMDA receptors was coupled in spinal dendrites, we transfected spinal neurons with the C terminus of GluR2 (GluR2CT). Previously, we have shown that the C terminus of GluR2 prevents the postsynaptic aggregation of AMPA receptors without affecting the formation of excitatory synapses themselves (Dong et al., 1997). As shown in Table 2 (panel A) and in the Supplemental Data (http://www.neuron.org/cgi/content/full/44/2/335/DC1/), GluR2CT but not the C terminus of GluR1 or the C terminus of NR2A significantly depressed the endogenous synaptic clustering of PSD-95 and Chapsyn-110 in spinal neurons. Moreover, GluR2CT but not the C terminus of GluR1 significantly reduced the NR2A-induced accumulation of synaptic NR1 in spinal neurons. These results imply that AMPA and NMDA receptor targeting are coupled at synapses.

Stargazin Is Present Only at Excitatory Synapses and Links Synaptic NMDA Receptors to AMPA Receptors in Spinal Neurons and Hippocampal Interneurons

Recent reports have shown that the tetraspanin stargazin and its related proteins can bind to both the MAGUK class of proteins (through its C terminus) and to AMPA receptors (via a site unrelated to the C terminus) providing a potential cytoplasmic link between AMPA and NMDA receptors through MAGUK proteins (Chen et al., 2000; El-Husseini et al., 2002). Moreover, these studies have also shown that a dominant-negative form of stargazin can be generated by deletion of its C-terminal PDZ binding domain, the portion responsible for coupling stargazin to MAGUK proteins (Chen et al., 2000). This mutant functions by disrupting interactions between NMDA and AMPA receptors. While Chen et al. and El-Husseini et al. demonstrated that stargazin mediated aggregation of AMPA-type glutamate receptors at synapses in hippocampal pyramidal cells, we felt that it might potentially work in reverse fashion at the AMPA predominant shaft synapses on spinal cord neurons and hippocampal interneurons.

As a first step in characterizing the role of stargazin in synaptic glutamate receptor aggregation in these cultures, we generated an antibody against the C terminus of rat

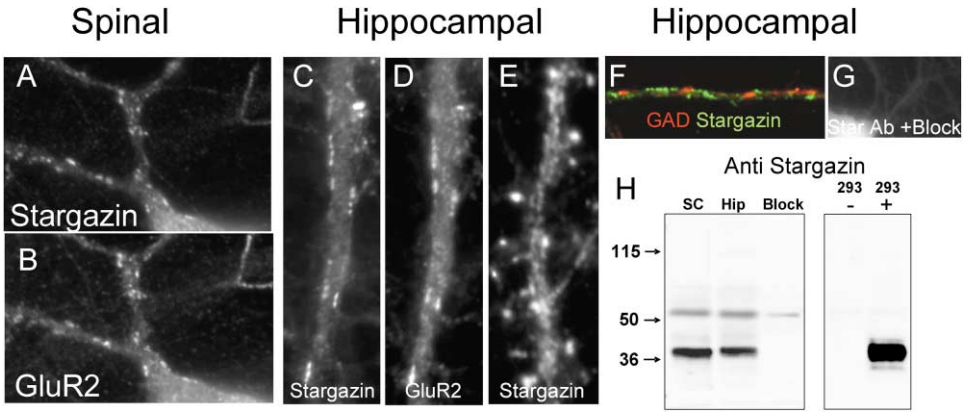

Figure 4. Stargazin Expression in Cultured Ventral Spinal and Hippocampal Neurons

C-terminal anti-stargazin peptide antibody shows one distinct, appropriately sized, peptide-blockable band in immunoblots of spinal and hippocampal neurons as well as in stargazin transfected HEK293 cells (H). This antibody shows peptide-blockable immunostaining that colocalizes with GluR2 in cultured ventral spinal neurons (A and B) and hippocampal neurons ([C]–[E]; day 10 in vitro). No colocalization of stargazin immunoreactivity was seen with GAD immunostaining (F).

stargazin, affinity purified it, and examined it on Western blots of cultured spinal and hippocampal neurons. As shown in Figure 4H, this antibody recognizes a protein of molecular weight 40 kDa, equally represented in both hippocampal and spinal neurons grown for 12 days in vitro. This is the same molecular weight as the signal from HEK293 cells transfected with recombinant human stargazin. A second minor band at molecular weight 55 kDa, seen in the cultured neurons, was not seen in stargazin-transfected HEK293 cells and was not blocked by preincubation with stargazin peptide. When used for immunostaining, the antibody showed peptide blockable signal only at excitatory synapses, colocalizing with AMPA receptors in both ventral spinal neurons (Figures 4A and 4B) and hippocampal (Figures 4C–4E) neurons. No stargazin immunosignal was seen to colocalize with the inhibitory synaptic marker GAD (Figure 4F). Of note, in hippocampal neurons, the stargazin signal was seen at excitatory synapses on both the shafts of hippocampal interneurons (Figures 4C and 4D) and the spines of pyramidal cells (Figure 4E). The appearance of stargazin at spinal synapses shows the same dependence on intact AMPA receptor targeting as does PSD-95 and Chapsyn-110, as it was disrupted by the dominant-negative GluR2 C-terminal tail (Table 2, panel A).

We transfected ventral spinal neurons with the dominant-negative truncated form of human stargazin, lacking its C-terminal four amino acids (designated stargazinΔC as per Chen et al., 2000), along with GFP to identify transfected neurons. Chen et al. (2000) have shown that this stargazin mutant fails to interact with endogenous MAGUK proteins, thus disrupting synaptic interactions. StargazinΔC (as well as the soluble C terminus of stargazin) inhibited the endogenous synaptic clustering of PSD-95 (and Chapsyn-110), similar to the effect of overexpression of the C terminus of GluR2 (Table 2, panel B).

In contrast to the effect of overexpressing the C terminus of GluR2, stargazinΔC did not affect GluR2 (or GluR1) synaptic clustering in ventral spinal neurons, either when calculated as the number of synaptic clusters of GluR2 per transfected neuron (Table 2, panel B) or when calculated as the mean synaptic GluR2 immunofluorescence per synapse (4877 +/− 967 units per synapse [Control] versus 5239 ± 1164 units per synapse [stargazinΔC]; mean ± SD for 86 and 91 synapses, respectively, in ten transfected neurons each). We also saw no change in the intensity of surface synaptic GluR1 clusters in stargazinΔC transfected neurons (1622 ± 298 units per synapse [Control] verus 2107 ± 401 units per synapse [stargazinΔC]; mean ± SD for 106 and 94 synapses, respectively, in ten transfected, GluR1-expressing neurons each). Transfection of full-length stargazin caused a modest increase in the number of synaptic GluR2 clusters per transfected neuron (Table 2, panel B) and a slight increase in the mean synaptic fluorescence intensity for GluR2 (4877 ± 967 units per synapse [Control] versus 5939 ± 1319 units per synapse [stargazin]; although this latter difference was not statistically significant).

In addition to its effect on the endogenous synaptic clustering of PSD-95, the stargazin mutants also blocked the NR2A-induced clustering of synaptic NMDA receptors in ventral spinal neurons (Table 2) and decreased the ability of exogenous Narp to cluster NMDA receptors on NR2A-transfected spinal neurons (see the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/]). This latter observation suggests that stargazin plays a similar role at Narp-induced receptor clusters and at synaptic receptor clusters. In order to ensure that a direct interaction between Narp, stargazin, and/or NR2A was not mediating synaptic NMDA receptor accumulation in cultured spinal neurons, we coexpressed stargazin and either Narp or NR2A in HEK293 cells. As shown in the Supplemental Data, no evidence was seen for coclustering or coimmunoprecipitation of Narp with either stargazin or NR2A (coexpressed with NR1).

Endogenous Synaptic NMDA Receptor Aggregation in Cultured Spinal Neurons

In order to investigate the discrepancy between our findings of no clustered synaptic NMDA receptors in cultured ventral spinal neurons and other reports suggesting the existence of synaptic NMDA receptors (Kalb

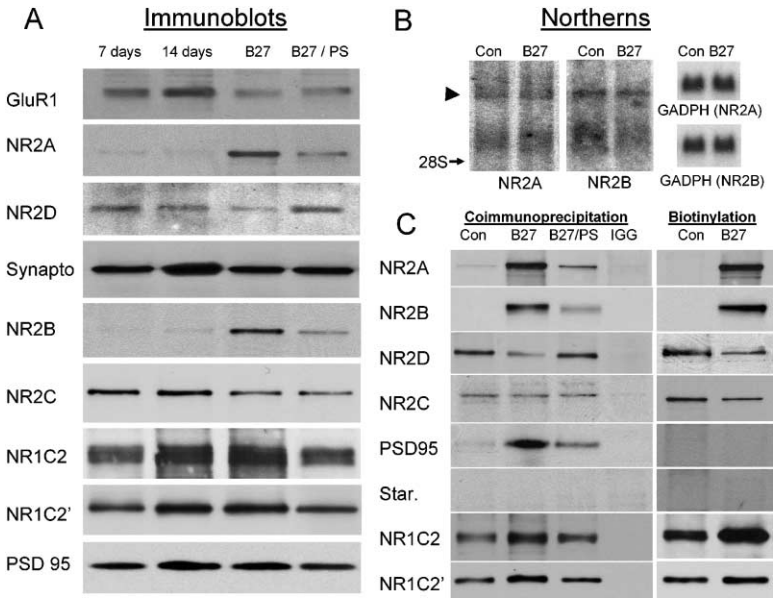

Figure 5. NMDA Receptor Subunit Expression Associated with Synaptic Accumulation

In (A), immunoblots of the indicated synaptic proteins were performed on high-density cultured ventral spinal neurons after 7 or 14 days in vitro or 2 days following a media switch to either Neurobasal-B27 (B27) or Neurobasal-B27 plus picrotoxin (100 μM) and strychnine (2 μM) (B27/PS). The media switch occurred between days 5 and 7 in vitro. Sister cultures had total RNA extracted for Northern blots of NR2A and NR2B mRNA levels (B) as described in Experimental Procedures. The arrowhead indicates the approximately 10–11 kb NR2A and NR2B transcripts, while the arrow shows the position of the 28S ribosomal RNA. In (C), NMDA receptors from spinal neurons grown for 7 days in control media or grown for 5 days in control media and then switched for 48 hr to B27 or B27 plus picrotoxin and strychnine (B27/PS) were immunoprecipitated using a combination of NR1C2 and NR1C2′ polyclonal antibodies covalently bound to Protein G (Seize-X, Pierce). The immunoprecipitate was probed with a series of synaptic proteins. A 2-fold excess of Rabbit IgG bound to the Seize-X beads served as control. Finally, surface proteins from 7 day control or B27 switched cultures were biotinylated using LC-Biotin (Pierce) and immunoprecipitated with streptavidin beads and probed with the indicated synaptic proteins. All experiments in Figure 5 were performed in triplicate, except the surface biotinylation experiment (5C), which was performed in duplicate.

et al., 1992; Konnerth et al., 1990; Ziskind-Conhaim 1990), we investigated conditions in our culture system that could lead to these discrepant results. Since we had always placed an emphasis on keeping motor neurons as healthy as possible, we routinely grew our cells in a very rich environment including chick embryo muscle extract and serum. In order to investigate whether these conditions were related to our lack of clustered synaptic NMDA receptors, perhaps by promoting excess synaptic activity, we switched our cultures after 5 days in vitro from their normal enriched media to a more minimal environment consisting of Neurobasal media with B27 supplements. As shown in Figure 5A, after 48 hr in this new media, both NR2A and NR2B protein levels could be seen to substantially increase, far past the level seen after 2 weeks in standard media. Quantitative densitometry showed a 6.8 ± 1.2-fold increase in NR2B and an 8.1 ± 2.4-fold increase in NR2A (n = 3).

Northern blots of total RNA from treated (B27) and untreated spinal neurons showed no change in NR2A or NR2B mRNA levels. The high molecular weight bands (approximately 10–11 kB; arrowhead, Figure 5B) corresponds to the size of the transcripts for NR2A and NR2B previously reported in the literature (Sasner and Buonanno, 1996; Desai et al., 2002) and showed no change in expression by densitometry in two separate experiments comparing control to B27-treated cultures (ratio of B27/Control = 0.92 ± 0.06 for NR2B and 0.94 ± 0.08 for NR2A; n = 2). The second band (smear) at approximately 6 kb (directly above the 28S ribosomal RNA band) likely represents a cross-reacting transcript or alternatively spliced transcript. It also showed no increase in level with media switch. Densitometry scanning of GAPDH showed that the same amount of total RNA was run in each lane.

Associated with the increase in NR2A and NR2B protein expression seen after media switch was the appearance of clustered synaptic NMDA receptors, consisting of both NR1 and NR2A (Figure 6 and Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/]). The effect of switching media could be antagonized by increasing the amount of ambient excitatory synaptic activity in the cultures grown in defined media by including picrotoxin and strychnine, GABA-A, and glycine receptor antagonist, respectively. As shown in Figures 5A and 6 and the Supplemental Data, the presence of picrotoxin and strychnine, which we have previously shown increases excitatory synaptic activity in these cultures (O'Brien et al., 1998), substantially abrogated the increase in NR2A and NR2B associated with media switching and significantly diminished the number and size of synaptic NMDA receptor clusters. It should be noted that the mean synaptic GluR2 immunofluorescence decreases with the same media switch that increases NR1 immunofluorescence and NR2A and -B protein levels, implying that this is not a general trophic effect.

In addition to the change in NMDA receptor subunit expression noted with the switch in growth media, we also observed a substantial change in the subunit composition of NMDA receptor complexes. As shown in Figure 5C, under basal conditions, immunoprecipitation of NR1 with a combination of NR1 C-terminal antibodies resulted in little coimmunoprecipitation of NR2A but substantial coimmunoprecipitation of NR2D. After switching to Neurobasal-B27, immunoprecipitation of NR1 resulted in coimmunoprecipitation of NR2A and NR2B along with lesser amounts of NR2D. The amount of NR2C immunoprecipitated with NR1 seemed unaffected by media change. These data are consistent with the results of immunostaining of extrasynaptic receptors seen in Figure 2, where extrasynaptic NR1 subunits (seen in standard culture conditions) appeared to be complexed with

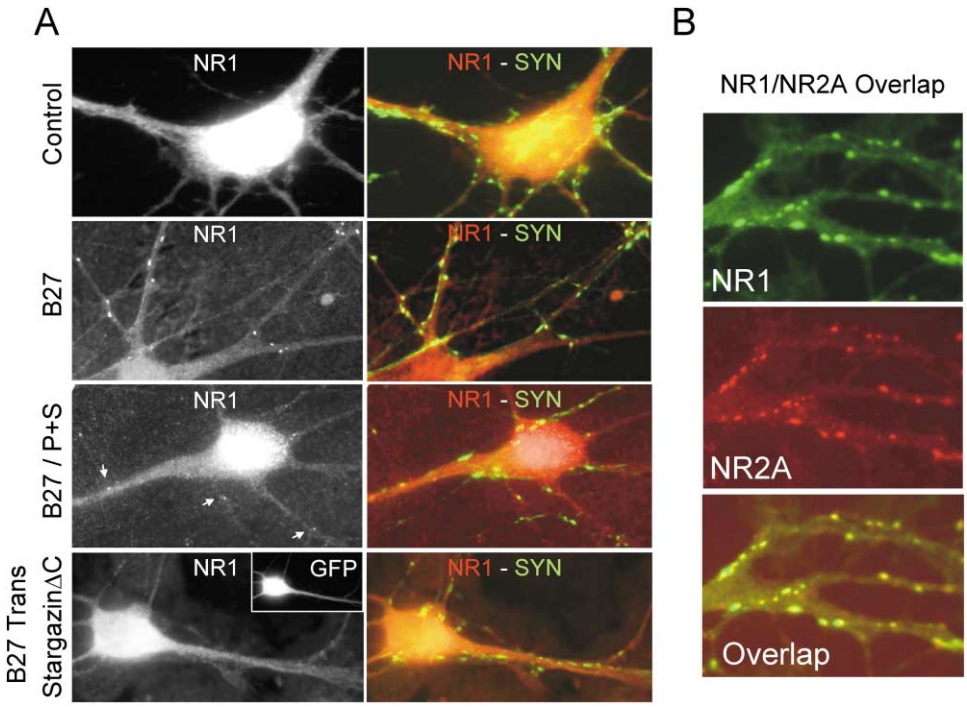

Figure 6. Ventral Spinal Neurons Demonstrate Synaptic NMDA Receptors after Switch to Defined Media

Ventral spinal neurons were grown for 5 days in standard growth media and then switched for 48 hr to either fresh growth media (Control), B27 media, or B27 media plus picrotoxin (100 μM) and strychnine (2 μM). Other cultures were switched to B27 after first being transfected with stargazinΔC and GFP. Synaptic NR1 clusters are seen most robustly in neurons switched to B27 media but were also seen in B27 plus picrotoxin and strychnine (B27/P + S) neurons. In the latter condition, the synaptic NR1 clusters (arrows) were smaller than those seen in B27 alone. Also noted in neurons grown in B27 media was a close correlation of NR1 with NR2A staining (B).

NR2D but not NR2A. Also consistent with the results of prior immunocytochemistry, surface biotinylation experiments (Figure 5C) showed that surface NR1 expression changes only marginally with the switch from culture conditions that promote a largely extrasynaptic population of NMDA receptors to one that promotes to a largely synaptic population.

In order to show that the expression of NR2A and NR2B was causally related to the synaptic clustering of NMDA receptors observed with the switch in culture conditions, we transfected a series of neurons after 5 days in culture (prior to media switch) with both GFP and the C-terminal tails of either NR1 (C2 and C2′), NR2A, or NR2D. In addition, we also evaluated the effect of the stargazin and GluR2CT dominant-negative mutants (see the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/]). Under these conditions, the C-terminal tail of NR2A but not the C-terminal tails of NR1 or NR2D was able to prevent the increase in synaptic NMDA receptor clustering induced by media switching. A similar effect was seen for the stargazin dominant-negative mutant (Figure 6A).

When the dominant-negative Narp mutant NarpN, which reduces the presynaptic secretion of endogenous Narp (O'Brien et al., 2002), is transfected into presynaptic axons under conditions in which spinal neurons express synaptic AMPA and NMDA receptors, the ability of presynaptic axons to cluster both AMPA and NMDA receptors as well as PSD-95 is reduced (see the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/]). The ability of these same NarpN transfected axons to cluster the inhibitory synapse-specific postsynaptic protein gephyrin is unaffected.

NMDA Receptor Aggregation in Hippocampal Neurons

To examine whether the native synaptic targeting of NMDA receptors in hippocampal neurons shares the features of NR2A-induced targeting in spinal neurons and to reconcile our results with those of Chen et al. (2000), we chose to examine the synaptic targeting of NMDA receptors in hippocampal interneurons. Like spinal neurons, hippocampal interneurons (which are largely GABAergic and identifiable by cytoplasmic GAD-65 staining in culture; Mi et al., 2002) cluster synaptic glutamate receptors on their dendritic shafts. These synaptic clusters of AMPA- and NMDA-type glutamate receptors on interneurons can be disrupted by the dominant-negative Narp mutant NarpN, which diminishes the secretion of Narp from presynaptic terminals (O'Brien et al., 1999; Mi et al., 2002). This contrasts with glutamate receptor clustering seen on the dendritic spines of hippocampal pyramidal cells in culture which is Narp (and NarpN) independent and developmentally deficient in AMPA receptors (Liao et al., 1999; Lissin et al., 1998).

We transfected hippocampal neurons with the C-terminal truncated stargazin dominant-negative mutant stargazinΔC on day 4 in vitro along with HcRed to identify transfected cells, replated them onto cortical astrocytes, and allowed them to grow an additional 5–7

Table 3. The Effect of StargazinΔC on GluR2 and NR1 Clustering in Hippocampal Neurons

Transfected at Replating with:	GAD-65+				GAD-65−			
	GluR2	NR1	PSD-95	Stargazin	GluR2	NR1	PSD-95	Stargazin
Control	13.6 ± 2.2	11.3 ± 2.2	—	—	—	17.6 ± 3.8	—	—
StargazinΔC	13.1 ± 2.4	4.8 ± 1.7**	—	—	—	19.3 ± 2.0	—	—
StargazinCTΔ10	15.9 ± 3.2	9.5 ± 1.9	—	—	—	19.1 ± 2.4	—	—
GluR2CT	5.7 ± 1.8**	4.1 ± 1.3**	—	—	—	14.8 ± 3.3	—	—
Transfected on Day 9 with:								
Control	34.0 ± 5.5	39.2 ± 6.4	36.8 ± 10.1	40.2 ± 7.0	29.9 ± 7.1	44.1 ± 7.8	38.8 ± 6.5	36.9 ± 4.8
StargazinΔC	29.8 ± 8.4	19.3 ± 5.3**	16.4 ± 7.5**	—	16.2 ± 5.1**	37.7 ± 12.1	31.6 ± 8.2	—
GluR2CT	18.3 ± 8.5**	25.3 ± 5.6**	21.1 ± 4.4*	27.1 ± 3.3**	17.4 ± 3.8**	40.3 ± 8.3	41.5 ± 5.2	38.8 ± 6.9

Hippocampal neurons were transfected with the indicated constructs plus a small amount of HcRed or GFP to identify the transfected cells, replated, and grown for 5–7 days, at which time they were fixed and stained for GluR2 or NR1 (mouse) and GAD (rabbit). A similar series of neurons were transfected on day 8 or 9 and examined on day 10–12. These neurons were stained either in the same manner described above or with stargazin (rabbit) and GAD (mouse) or with PSD-95 (mouse) plus GAD (rabbit). The numbers expressed are the mean ± SD of the mean of three separate experiments. For each experiment, 20 to 25 consecutive, randomly selected HcRed/GFP-positive neurons (both GAD+ and GAD−) were chosen and the number of GluR2, NR1, stargazin, or PSD-95 clusters counted in one field (cell centered) at 100×. Cells that had no clusters of GluR2 (approximately 20% of the GAD 65+ neurons transfected with each of the three constructs) were not counted. The slides were blinded before counting. AMPA receptor clusters were not counted on young GAD65− neurons (presumptive pyramidal cells) because it was difficult to be certain of discreet clustering of these receptors at this stage. Synaptic NR1 clustering was, however, robust at this time. Neurons transfected with stargazinΔC were not stained for stargazin, because of antibody cross-reaction. **$p < 0.02$ compared with control using paired Student's t test; *$p < 0.05$.

days. Interneurons (identified by their blue cytoplasmic staining with the polyclonal GAD 65 antibody) showed no change in AMPA receptor clusters but a significant decrease in NMDA receptor clusters when transfected with stargazinΔC (Table 3; see also the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/]). In contrast, neurons which were GAD 65 negative had no change in the number of NMDA receptor clusters.

We also transfected more mature hippocampal neurons on day 9 in vitro, without replating, and examined them 48–72 hr later, using permeablized GluR2, NR1, stargazin, and PSD-95 staining (Table 3). The data demonstrate a differential effect of stargazin and GluR2 mutants on glutamate receptor clustering in interneurons versus pyramidal neurons. In interneurons, synaptic NR1 clustering is dependent on stargazin and AMPA receptor localization, determined by their respective dominant-negative mutants, whereas in pyramidal neurons synaptic NR1 localization is resistant to stargazinΔC and GluR2CT disruption. Furthermore, native stargazin immunostaining is disrupted by GluR2CT in interneurons, while in pyramidal neurons GluR2CT had little effect on stargazin localization, perhaps indicating that stargazin is more a scaffolding protein in these cells. Older neurons were more resistant to the effects of the mutants than younger neurons, possibly due to changes in receptor turnover rates. The lack of change of PSD-95 clusters in hippocampal pyramidal neurons transfected with any of the constructs argues against a change in the number of excitatory synapses per se.

Discussion

NR2A and -B Expression Cause Synaptic Clustering of NMDA Receptors

In this paper, we have shown that ventral spinal neurons, deficient in the expression of the NMDA receptor subunits NR2A and NR2B, can have synaptic NMDA receptor targeting reconstituted (as determined by NR1 immunostaining) by the overexpression of these same subunits. Overexpression of NR2C, NR2D, NR1, PSD-95, or SAP-102 had no effect on the synaptic targeting of NMDA receptors. These results offer an explanation for the lack of basal NMDA receptor clustering at synapses in ventral spinal neurons (O'Brien et al., 1997; Mi et al., 2002) and make this system extremely useful for evaluating how NMDA receptors become aggregated at synapses. The importance of the NR2 subunits in the synaptic targeting of NMDA receptors has been previously demonstrated by Barria and Malinow (2002), who showed that NR2A or NR2B aided the synaptic delivery of cotransfected NR1 in hippocampal slice neurons. The main difference between our observation and theirs is that cultured ventral spinal neurons do not have endogenous synaptic NMDA receptors prior to transfection with NR2A or NR2B, either physiologically (O'Brien et al., 1997) or immunohistochemically (O'Brien et al., 1997, 1998), while synaptic NMDA receptors are already present prior to transfection in hippocampal slice preparations. Therefore, our data show that NR2A and -B not only facilitate synaptic insertion but can initiate it. Moreover, our results indicate that the synaptic targeting motifs on NR2 involve both the extreme C terminus, the site of the PDZ binding domain, and at least one other site. The last six amino acids of NR2A and NR2B are identical and differ from NR2C and NR2D at two amino acids. These differences, although involving physically similar amino acids, results in a significant alteration in the interaction with PSD-95 and Chapsyn-110. Previously, Mori et al. (1998) and Steigerwald et al. (2000) found that C-terminal truncation mutants of NR2A and -B decreased synaptic targeting of NMDA receptors in vivo, while Sprengel et al. (1998) did not observe this. The overlapping function and distribution of NR2A and -B, both of which can direct synaptic targeting of NMDA receptors, is possibly the explanation for the failure to

see effects of single mutants and underlines the importance of in vitro systems for dissecting these effects. Overexpression of PSD-95 did not increase the synaptic targeting of NMDA receptors in spinal neurons grown in any culture conditions. One explanation for this is that PSD-95 and/or Chapsyn-110 may be expressed in saturating concentrations under basal conditions. Unlike Barria and Malinow, we did not see a significant effect of synaptic activity on NR2A or -B mediated synaptic targeting, likely reflecting differences in the characteristics of the underlying cell types or possibly in the levels of expression of the transfected construct.

The existence of a second motif on NR2A and NR2B necessary for synaptic targeting of NMDA receptors was suggested from the chimeric NR2A/D experiments. It is unlikely that this motif mediates the assembly of a multimeric receptor complex, as our data clearly show that NR2D associates with NR1 in an extrasynaptic multimeric surface receptor complex. Moreover, we have found no evidence that NR2A associates directly with Narp or stargazin. Additional C-terminal or N-terminal interactions seem the most attractive possibilities and will be further explored using chimeric receptors. Recent work with both Narp (O'Brien et al., 1998) and GluR2 (Passafaro et al., 2003) has established the precedence of extracellular determinants on glutamate receptors as synaptic targeting motifs.

Finally, a recent report by Mu et al. (2003) showed that, in hippocampal neurons, the C2′ cassette of NR1, downregulated under basal conditions, leads to synaptic accumulation of NMDA receptors when overexpressed or upregulated due to synaptic blockade. We have found that NR1 subunits expressing this cassette are plentiful under basal conditions in cultured ventral spinal neurons, conditions in which synaptic NMDA receptors are lacking. Moreover, overexpression of NR1-4A, which contains the C2′ cassette, does not increase synaptic NMDA receptor targeting. These and other studies underline the important differences between spinal and hippocampal neurons, a difference we believe arises from the shaft-predominant synapses in the spinal neurons and the spine-predominant synapses in hippocampal neurons.

Culture Conditions Alter Synaptic NMDA Receptor Accumulation

Switching spinal neurons from a growth factor-rich environment to a defined media caused both the appearance of NR2A and NR2B and the synaptic accumulation of NMDA receptors, events that our dominant-negative constructs suggest are causally related. Associated with this is a decrease in GluR1 expression and a diminution of synaptic AMPA receptor accumulation. The NR2A or NR2B accumulation occurred in the absence of any change in mRNA expression, reminiscent of the effect of synaptic activity on synaptic AMPA receptor accumulation (O'Brien et al., 1998), and possibly implicates protein turnover as the causative mechanism for subunit expression levels. Precedents for growth factor effects on glutamate receptors exist. Rutherford et al. (1998) found that BDNF significantly modified AMPA receptor-mediated quantal amplitude in hippocampal pyramidal neurons (with an opposite effect on hippocampal interneurons), and Choi et al. (2004) have shown NR2A induction by neurotrophin 4/5 in cultured neurons. The effect we see is possibly a result of both direct growth factor action and alterations in synaptic activity due to the changing environment.

NMDA Receptors Are Coupled to AMPA Receptors

Our data indicate that synaptic NMDA receptor aggregation is dependent on the integrity of the AMPA receptor system. As such, conditions that disrupt AMPA receptor accumulation at excitatory synapses, such as overexpression of the C terminus of GluR2, also disrupt the synaptic accumulation of MAGUK proteins and associated NMDA receptors. Given that the C terminus of GluR2 likely plays a role in transport and surface localization of AMPA receptors (Dong et al., 1997; Burette et al., 2001; Sheng and Lee 2003) but not in any interaction with Narp (extracellular), stargazin (transmembrane), or PSD-95 (no proven interaction), its effect on NMDA receptors is likely to be mediated purely through the failure of AMPA receptors to appear at excitatory synapses. The failure of the C terminus of GluR1 to affect AMPA receptor trafficking in spinal neurons is in contrast with results in hippocampal neurons where acute activity-induced synaptic redistribution of AMPA receptors (as seen in LTP) is mediated by GluR1, while constitutive synaptic redistribution is modulated by the C terminus of GluR2 and GluR3 (See Lee et al., 2004). Given that acute activity-dependent redistribution of AMPA receptors is difficult to demonstrate in neurons with predominantly shaft synapses (McBain et al., 1999), it may be that spinal neurons and hippocampal interneurons lack the GluR1-mediated component of receptor cycling. Alternatively, the activity-induced cycling of GluR1 may modulate GluR1 accumulation but not that of other AMPA or NMDA receptor subunits.

Previously, we have shown that the dominant-negative Narp mutant NarpN disrupted AMPA receptor aggregation at hippocampal interneuron synapses and also disrupted synaptic NMDA receptor accumulation. Given that Narp has no endogenous ability to interact with NMDA receptors (O'Brien et al., 1999; Mi et al., 2002), we felt that this indicated that the failure of NMDA receptors to aggregate at synapses was secondary to the failure of Narp-dependent AMPA receptor aggregation. The same effect of NarpN on AMPA and NMDA receptor clustering is seen in spinal neurons grown in defined media. This conclusion is further strengthened by the observation that stargazin mutants disrupt PSD-95/NMDA receptor aggregation without affecting AMPA receptor aggregation in spinal neurons and hippocampal interneurons. That NarpN, stargazinΔC, and GluR2CT are not toxic is shown by the lack of effect of these mutants on NMDA receptor aggregation in hippocampal pyramidal cells (see below).

Our data would also argue against a system in which NMDA receptors are guided by AMPA receptors only through their intracellular transport and surface insertion phase. Using epitope-tagged NR1, we saw no influence of GluR2CT on surface NR1 insertion and observed that NR1 surface expression (defined by surface biotinylation) is quite robust even when the receptors are almost exclusively extrasynaptic. Finally, the ability of Narp trans-

fected COS cells to aggregate NMDA receptors on NR2A transfected spinal neurons (and hippocampal interneurons; Mi et al., 2002) demonstrates the sufficiency of Narp alone for this process. Since Narp does not aggregate NMDA receptors directly, even when coexpressed on the same HEK293 cell (Mi et al., 2002, and the Supplemental Data [http://www.neuron.org/cgi/content/full/44/2/335/DC1/]), a direct linkage between AMPA receptors and NMDA receptors, independent of additional presynaptic aggregating molecule, appears to be the best explanation.

Experiments using chimeric NR2A/NR2D molecules suggest an additional NR2A- or NR2B-mediated link to excitatory synapses through a motif located outside the extreme C terminus. Whether this additional NR2A or NR2B motif facilitates AMPA-NMDA receptor interactions or serves as a link to an additional synaptic organizing molecule(s) will await further work.

MAGUK Protein and Stargazin Expression in Cultured Ventral Spinal Neurons

The mechanism of linkage between NMDA and AMPA receptors appears to be dependent either on stargazin itself, or a closely related molecule (Tomita et al., 2003), and a MAGUK protein. Both the soluble stargazin C-terminal tail and the transmembrane component of stargazin without its C-terminal tail act in a dominant-negative fashion toward NMDA receptor and MAGUK protein synaptic aggregation in spinal neurons and hippocampal interneurons. This indicates that it is not simply a molecule that shares the C-terminal PDZ binding motif of stargazin that is responsible for coupling the receptor types but a molecule which shares the AMPA receptor-interacting domain of stargazin as well. Immunostaining with stargazin-specific antiserum indicates that stargazin is in place at AMPA-predominant synapses in spinal and hippocampal neurons, extending the prior findings of Sharp et al. (2001). The PDZ binding sites of NR2A and NR2B (IESDV) likely bind to PDZ 1 and -2 of PSD-95 (Hung and Sheng, 2002). The different PDZ binding domain of stargazin (RTTPV) is similar to that of neuroligin (TTPV) and likely binds to PDZ 3 of PSD-95. As such, overexpression of the C-terminal tail of NR2A and NR2B would not be expected to affect the synaptic targeting of PSD-95 or Chapsyn-110, which is indeed the case (Table 2).

Given the nonsynaptic localization of NMDA receptors in cultured ventral spinal neurons, we were initially surprised to see synaptic clustering of PSD-95 and Chapsyn-110/PSD-93 under basal conditions. However, given the presence of stargazin at these synapses under basal conditions, it is not, in retrospect, surprising to find colocalized MAGUK proteins. Other investigators have established an interaction between stargazin and PDZ domain-containing proteins though stargazin's C terminus (Schnell et al., 2002). The recent finding that Chapsyn-110/PSD-93 knockout mice show impaired synaptic NMDA receptor expression but normal AMPA receptor expression (Tao et al., 2003) is in agreement with our observations. Whether Chapsyn-110 or PSD-95 serve identical/overlapping functions or have subtly different functions or distributions is not clear. Our data suggest that the synaptic localization of both is tied to stargazin or a similar molecule. Given the dual expression of PSD-95 and Chapsyn-110 at spinal synapses, it is likely that double knockouts will be necessary to completely evaluate the role of MAGUK proteins in the synaptic organization of spinal neurons.

Hippocampal Interneurons Differ from Pyramidal Neurons in their Mechanism of Synaptic NMDA Receptor Targeting

Our current data again emphasize the differences between excitatory synapse formation on hippocampal interneurons and pyramidal neurons. In addition to differences in their neurotransmitter phenotype (interneurons are predominantly inhibitory while pyramidal cells are predominantly excitatory), they also differ in the manner in which they receive excitatory synapses on their dendrites. Spinal cord neurons share many of the features of hippocampal interneurons, although their neurotransmitter phenotype is more variable. Hippocampal interneurons and spinal neurons receive excitatory synapses directly onto their dendritic shafts, while pyramidal cells receive excitatory synapses onto their dendritic spines. In addition to differences in morphology, synaptic proteins also appear to be differentially expressed at excitatory spine and shaft synapses. Examples include SynGAP, citron, bundled actin filaments, and Narp (Allison et al., 1998; Zhang et al., 1999; Mi et al., 2002). Even NMDA- and AMPA-type glutamate receptor subunits are themselves expressed differentially at these two types of excitatory synapses (O'Brien et al., 1997; Allison et al., 1998; Gomperts et al., 1998; Lissin et al., 1998; Rao et al., 1998; Liao et al., 1999). Functionally, excitatory synapses on spines and shafts also differ. Dendritic shaft synapses in hippocampal interneurons have been resistant to LTP induction (McBain et al., 1999) and undergo homeostatic scaling in a manner different from pyramidal cells (Rutherford et al., 1998).

Our observations of a fairly uniform accumulation of synaptic NMDA receptor clusters in hippocampal interneurons are somewhat at variance with those of Nyiri et al. (2003), who found significant differences in synaptic NMDA receptor accumulation between different classes of interneurons in vivo. Given the differences between our in vitro system and their in vivo system, the source of these differences could be either the selection of a single class of interneurons for culture related to the staged birth of interneuron classes in vivo (E18–21) or could reflect a leavening effect of tissue culture conditions.

Consistent with the ongoing emphasis on the differences between interneurons and pyramidal cells, our results contrast with those of Chen et al. (2000), who found that the dominant-negative mutant stargazinΔC only affected AMPA receptor clustering in hippocampal neurons. We feel that this difference is likely the result of the predominance of pyramidal cells ($>$85%) in hippocampal cultures. We also found no effect of stargazinΔC on NMDA receptor clustering in these cells. It will be of great interest to see if the differences between these two cell types are due to relative mobility issues between AMPA and NMDA receptors, perhaps mediated by the pattern of MUGAK protein expression.

A useful model of synaptic glutamate receptor local-

ization in spinal neurons and hippocampal interneurons would involve AMPA receptor transport/synaptic delivery mediated by cytoplasmic factors that bind to the C terminus of GluR2 (and GluR3). These receptors could then be stabilized at the synapse (once inserted) by Narp. Stargazin would play a bridge role to synaptic NMDA receptors and less of a role in synaptic AMPA receptor localization. In hippocampal pyramidal cells, cytoplasmic AMPA receptor interactions are still important, but stargazin assumes a more significant role in the synaptic delivery of AMPA receptors and less of a role in synaptic NMDA receptor localization. Narp is absent at these synapses, although other synaptic organizing molecules that bind directly to AMPA receptors may be present (Xu et al., 2003; Passafaro et al., 2003). Synaptic aggregating molecules such as the neurexins and ephrins could couple directly to PSD-95 without an AMPA receptor bridge, allowing them to aggregate NMDA receptors without an AMPA receptor intermediary (Gerlai, 2001; Scheiffele et al., 2000). These would be ideally situated to regulate spiny synapses, but whether they are differentially regulated at spine and shaft synapses has not been established.

Experimental Procedures

Cell Culture

Low- and high-density ventral spinal neurons (from E15 rat embryos) and hippocampal neurons (from E18–20 rat embryos) were isolated and grown as described (O'Brien et al., 1997) on a bed of preplated cortical glia. Growth media consisted of MEM plus N2 supplements as well as E18 chick hindlimb extract at 15 μg/ml (Henderson et al., 1993; O'Brien et al., 1997) and 3% horse serum. Cultures were maintained from 7 to 17 days, depending on the experimental paradigm, with every other day feedings of 1/3 the culture volume. In some cultures, growth media was switched after 5 days to a media consisting of Neurobasal (Invitrogen) plus B27 supplements (1:50). This media was supplemented with glutamax 1:200 and was conditioned by a monolayer of glia for 8 hr prior to use.

Constructs

See the Supplemental Data (http://www.neuron.org/cgi/content/full/44/2/335/DC1/).

Neuronal Transfections

Spinal and hippocampal neurons were routinely transfected using lipofectamine 2000 (Invitrogen) after 3 days in vitro by combining 1 μg of total DNA with 2 μl of lipofectamine 2000 in a final volume of 70 μl. This solution was added to one well in a 12-well dish containing 30,000 neurons and 800 μl of MEM for 90 min at 37°C. The cells were then rinsed in MEM and their original media added back. Four hours after transfection, the cells in each well were trypsinized off the plate with 100 μl of 0.025% trypsin, mixed with 1 ml of fresh growth media, and added to a fresh well containing a glial-coated coverslip. After 12 hr, the media was completely changed, and the cells were then fed regularly for 4–7 days until assayed. Additional hippocampal cultures were transfected after 8 or 9 days in vitro and examined (without replating) after an additional 48–72 hr. Similarly, in some cases, spinal neurons were transfected with various constructs at 5 days in vitro without replating, immediately prior to switching the growth media to Neurobasal plus B27.

Immunohistochemistry

Neuronal cultures were fixed in 4% paraformaldehyde, 4% sucrose in PBS for 5 min, transferred to methanol at −20 for an additional 15 min, permeablized in 0.1% Triton X-100 for 10 s, and then rinsed twice in PBS. Coverslips were then blocked in 10% goat serum at room temperature for 1 hr and incubated with primary antibodies for 24 hr at 4°C. Rhodamine, AMCA, or fluorescein-conjugated secondary antibodies (Jackson Immunochemicals, West Grove, PA) were then added at 10 μg/ml for 1 hr at room temperature as indicated. Coverslips were mounted in ProLong (Molecular Probes). Live immunostaining prior to fixation was carried out for 30 min at 37°C in MEM plus APV (100 μM) and CNQX (10 μM).

In experiments looking at the colocalization of NR1 and NR2D or NR2A immunosignal in extrasynaptic receptors, *myc*-NR1 transfected neurons were stained live with mouse monoclonal anti-*myc*, fixed in sequential paraformaldehyde and methanol, and then stained with either polyclonal NR2A or NR2D and goat anti-synaptophysin (Santa Cruz). In order to perform a block of NR2D staining, HEK293 cells were transfected with NR2D (or NR2A) along with NR1. The cells were then surface biotinylated, solublized, and the surface-labeled proteins streptavidin immunoprecipitated. The labeled beads from NR2D transfected cells were incubated with the NR2D antisera in PBS for 1 hr at 4° and then used directly to stain cells. NR1/NR2A transfected HEK293 cells served as a negative control for the NR2D antibody to ensure that we were specifically absorbing antibody.

Clustering Assay

At variable times after transfection, neuronal coverslips were fixed and stained as described above. In most cases, the number of synaptic clusters of a given protein was assayed by combining rabbit and mouse antibodies to synaptic proteins (i.e., GluR1 or NR1) with mouse or rabbit synaptophysin antibodies, respectively. The technical details of the assay, its blinding, and how it is scored have been published (OBrien et al., 2002). In all cases, except for assays of quantitative synaptic immunofluorescence, the numbers expressed are the mean ± SD of the mean of three or four separate experiments, each run with a control condition. The semiquantitative estimation of extrasynaptic clusters was described in Mammen et al. (1997). Quantitative estimates of synaptic NR1 and GluR2 accumulation were performed as described (O'Brien et al., 1999) and in each case involved at least 200 randomly chosen synapses from 20 neurons over two platings unless otherwise specified. If the neuron had a mixture of synapses that appeared both immunopositive and immunonegative (as in the case of NR2A/D6 transfection) only the synapses that were deemed immunopositive were analyzed. Thus, this technique places an upper limit on the mean synaptic immunofluorescence. Experiments in hippocampal neurons involving GAD-positive and -negative neurons did not use synaptic clusters per se, given the lack of chromophores. The efficacy of NarpN and control transfected axons to cluster GluR2, PSD-95, and gephyrin on untransfected postsynaptic dendrites was assayed as described in O'Brien at al. (2002). For this latter assay, a minimum of 80 transfected axons (control and NarpN) were examined for each postsynaptic protein assayed over three platings.

Antibodies

A C-terminal stargazin antibody was raised in rabbits by immunizing them with the rat stargazin C-terminal peptide KDSKDSLHAN TANRRTTPV coupled to albumin. The resultant serum was purified on an affinity column of the same peptide coupled to thyroglobulin. The affinity-purified antibody was used at 1 μg/ml for immunoblots and at 2 μg/ml for immunostaining. Antibodies to the NMDA receptor subunit NR2C was obtained from Molecular Probes and used at 1:300 for immunoblots and staining, while the NR2D antibody was purchased from Santa Cruz and used at 1:100 for staining and 1:200 for immunoblots. NR2A antibodies were obtained from Upstate Biotechnology (polyclonal #06313), raised against amino acids 1260–1460 of mouse NR2A and used in immunoblots at 1:600 and for immunostaining at 1:300, and from Chemicon (MAB5216), raised against amino acids 1099–1213 of human NR2A. The Chemicon NR2A antibody was used at 3 μg/ml for immunoblots. NR2B antibodies were obtained from Transduction Labs (monoclonal #610416), raised against rat amino acids 892–1051 and used at 1 μg/ml for immunoblots and 2 μg/ml for immunostaining and a rat extreme C-terminal peptide polyclonal anti NR2B antibody (Lau and Huganir 1995) used at 2 μg/ml for immunoblots and 5 μg/ml for immunostaining. NR1 antibodies consisted of the C2′ cassette-specific polyclonal antibody from Pharmingen (#690KC; used at 2 μg/ml for immunoblots) and the C2 cassette-specific polyclonal antibody from Upstate Biotechnology

(#06311: used at 1 μg/ml for immunoblots). For NR1 immunostaining, we used the S3C11 pan NR1 monoclonal. The PSD-95 monoclonal antibody was obtained from Transduction Labs (#610495) and used at 1 μg/ml for immunoblots and at 5 μg/ml for immunostaining. We also used the anti PSD-95 monoclonal from Upstate (#05-494) with similar results.

The Chapsyn-110 polyclonal was purchased from USBiological and used at 1:300 (immunoblots) and 1:200 (immunostaining). Antibodies against the *myc* epitope, GluR2 (monoclonal), GluR2/3 (polyclonal), tubulin, synaptophysin (mouse and rabbit), and GAD (mouse and rabbit) have been described in our previous publications (O'Brien et al., 1997, 2002), as have the SAP-97 (Rumbaugh et al., 2003) and SAP-102 antibodies (Lau et al., 1996).

Coimmunoprecipitation Experiments: HEK293 Cells

HEK293 cells were transfected with N-terminal *myc*-tagged versions of the C terminus of NR2A (30 kDa), NR2D (16 kDa), and PSD-95 with a DNA ratio of 1, 2, 1, respectively. Additional cells were transfected with the C-terminal constructs NR2A/D6, NR2D/A6, and PSD-95 in a 2, 1, 1 DNA ratio. A similar set of experiments was done using Chapsyn-110 rather than PSD-95. The cells were solublized in PBS plus 1% Triton X-100 plus protease inhibitors (Pierce). PSD-95 was immunoprecipitated with mouse anti-PSD-95 (UBI 05-494) followed by protein A/G beads (Pierce), washed twice with PBS plus 0.2% Triton X-100, and once in PBS plus 200 mM NACL. The total input (1/20 of the total material) and the immunoprecipitate (1/3 of the total immunoprecipitate) were probed with rabbit anti-*myc* (Covance) on a 12% acrylamide gel. Chapsyn-110 was immunoprecipitated with a polyclonal anti-Chapsyn antibody (USbiological), and a mouse anti-*myc* antibody (Upstate) was used to probe the gels. A similar protocol was used for the coimmunoprecipitation of *myc*-Narp or *myc*-stargazin with either GluR1, NR1/N22A, stargazin, or Narp except that the solublized cells were immunoprecipitated with mouse anti-*myc* monoclonal. In these experiments, the *myc*-tagged Narp or stargazin construct was transfected with the additional construct at a 1:1 DNA level, except when the combination of NR1/NR2 was used, in which case the ratio was 1, 1, 1 *myc*-Narp, NR1, NR2A.

Coimmunoprecipitation Experiments: Neurons

Ventral spinal neurons grown at high density in 10 cm dishes either in routine media or in media changed after 5 days to Neurobasal plus B27 were solublized in 2 ml of PBS plus 1% Triton X-100 plus protease inhibitors (Pierce) followed by brief sonication. NMDA receptor complexes were immunoprecipitated with Seize X beads to which a combination of NR1 antibodies were bound. In brief, 100 μg of polyclonal anti-NR1 antibodies consisting of an equal mixture of C2′ (Sigma) and C2 (Upstate Biotechnology) cassette-specific antibodies were covalently linked to a 0.3 ml slurry of Protein G using DSS (disuccinimidyl suberate) per the manufacturer's instructions (Pierce). Control consisted of 200 μg of preimmune rabbit IGG linked to a 0.3 ml slurry of Protein G. Active or control beads (50 μl) were incubated with the solublized protein from each 10 cm dish for 4 hr at 4°, washed twice with PBS plus 0.2% Triton X-100, and once in PBS plus 200 mM NACL and then solublized in 200 μl of 1× sample buffer. Immunoprecipitate (40 μl) from each experimental condition was then run on a 10% acrylamide gel and immunoblotted with antibodies. The Seize-X beads allowed us to use rabbit antibodies to immunoprecipitate and immunoblot without contaminating IGG bands.

Immunoblotting

High-density cultures of hippocampal or ventral spinal neurons at a similar density were grown for 12 days in vitro. Cultures were solublized in equal volumes of M-Per plus protease inhibitors (200,000 cells per ml) and run on SDS PAGE, transferred to Immobilon-P (Millipore Corp), probed with the indicated antibodies, and visualized with enhanced chemiluminescence (ECL, Amersham Corp.). All experiments were done in triplicate and yielded similar results. Prior standardization has indicated that there was little cell death in these cultures, making volumetric equivalency a more accurate indication of per-cell differences. Separate experiments using similar amounts of protein from each well yielded similar results.

Surface Biotinylation

Cell surface proteins were biotinylated and streptavidin immunoprecipitated as described in Mammen et al. (1997). Equivalent volumes of material from control and Neurobasal B27-treated cultures were run, each lane representing 1/10 of the total surface material from a 10 cm dish of neurons.

Northern Blots

High-density 10 cm dishes of spinal neurons were grown in standard media or switched for 48 hr to Neurobasal media plus B27 supplements. Total RNA was extracted using Totally RNA (Ambion) and processed for Northern blotting as described (O'Brien et al., 1998), running 5 μg of RNA per lane. Probes consisting of a 1.2 kb Pst1 fragment of rat NR2A and an 800 bp PCR fragment (3677–4470) of rat NR2B were labeled with ^{32}P by random priming. Blots were exposed to a phosphorimager for 9 days and quantitated using ImageQuant. Blots were stripped and reprobed with a 500 bp fragment of rat GAPDH.

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